

RIBOSOMAL DNA PHYLOGENY OF MARINE ANAMORPHIC FUNGI: *CUMULOSPORA VARIA*, *DENDRYPHIELLA* SPECIES AND *ORBIMYCES SPECTABILIS*

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ABSTRACT. – The marine anamorphic fungi, *Cumulospora varia*, *Dendryphiella arenaria*, *Dendryphiella salina* and *Orbimyces spectabilis*, have no known teleomorphs, and DNA sequence analyses were undertaken to determine their ordinal classification and putative teleomorphs. Molecular phylogenetic analyses of the combined SSU+LSU and ITS rDNA datasets were used to resolve the relationships between terrestrial (*Dendryphiella vinosa*) and marine *Dendryphiella* species (*Dendryphiella arenaria* and *Dendryphiella salina*), a genus with macronematous conidiophores, polytretic conidiogenous cells and dematiaceous phragmoconidia. The combined SSU+LSU rDNA dataset indicates that both marine *Dendryphiella* species cluster in the order Pleosporales and family Pleosporaceae. In analyses of the ITS rDNA dataset they form a sister clade to the *Pleospora herbarum*/*Stemphylium* species complex. *Dendryphiella salina* CY3139 clustered with another isolate CY3140, together with *D. arenaria*, indicating that marine *Dendryphiella* species are monophyletic. The evolutionary lineage of marine *Dendryphiella* species is closely related to that of the genera *Pleospora* and *Stemphylium*. Phylogenetic analysis of the SSU+LSU rDNA of *Cumulospora varia* and *Orbimyces spectabilis* indicates that their putative teleomorphs are to be found in the Lulworthiales. *Cumulospora varia* forms a sister group with *Lulwoana uniseptata* and its anamorph *Zalerion maritimum*, while *Orbimyces spectabilis* groups with *Lulwoidea lignoarenaria*.

KEY WORDS. – Lulworthiales, marine anamorphic fungi, molecular phylogeny, ordinal assignment, Pleosporales, taxonomy.

INTRODUCTION

Marine anamorphic fungi total 88 species in 54 genera, while marine ascomycetes account for 417 species in 204 genera, but only 37 teleomorph/anamorph connections have been established between the marine taxa (Chatmala et al., 2002). Most of these connections have been made by culture techniques (Shearer & Crane, 1977; Nakagiri, 1984, 1986; Mouzouras & Jones, 1985) while a few have resulted from molecular studies (Bills et al., 1999; Mantle et al., 2006).

The genus *Cumulospora* was described with *Cumulospora marina* as the type species [as *Vesicularia*, previously preoccupied by earlier homonyms in Schmidt (1974)] (Schmidt, 1985), and the second marine species, *Cumulospora varia*, was described by Chatmala et al. (2004). As it predates

the earlier genus, *Basaramyces* is a synonym of *Cumulospora* (Abdullah et al., 1989). Conidia of *Cumulospora marina* are larger (42–82 × 42–85 μm) than those of *Cumulospora varia* (52–91 × 40–71 μm) (Fig. 1) and differ in their morphology.

The type species of *Dendryphiella* is *D. vinosa*, a terrestrial fungus with the following morphological features: conidiophores macronematous bearing conidia at the swollen tip and at intercalary swellings, polytretic, integrated, terminal but becoming intercalary, sympodial, cicatrized on the nodose swellings with scars close to each other; conidia cylindrical with rounded ends, dematiaceous, multi-septate, catenate or solitary, acropleurogenous (Kohlmeyer & Kohlmeyer, 1979).

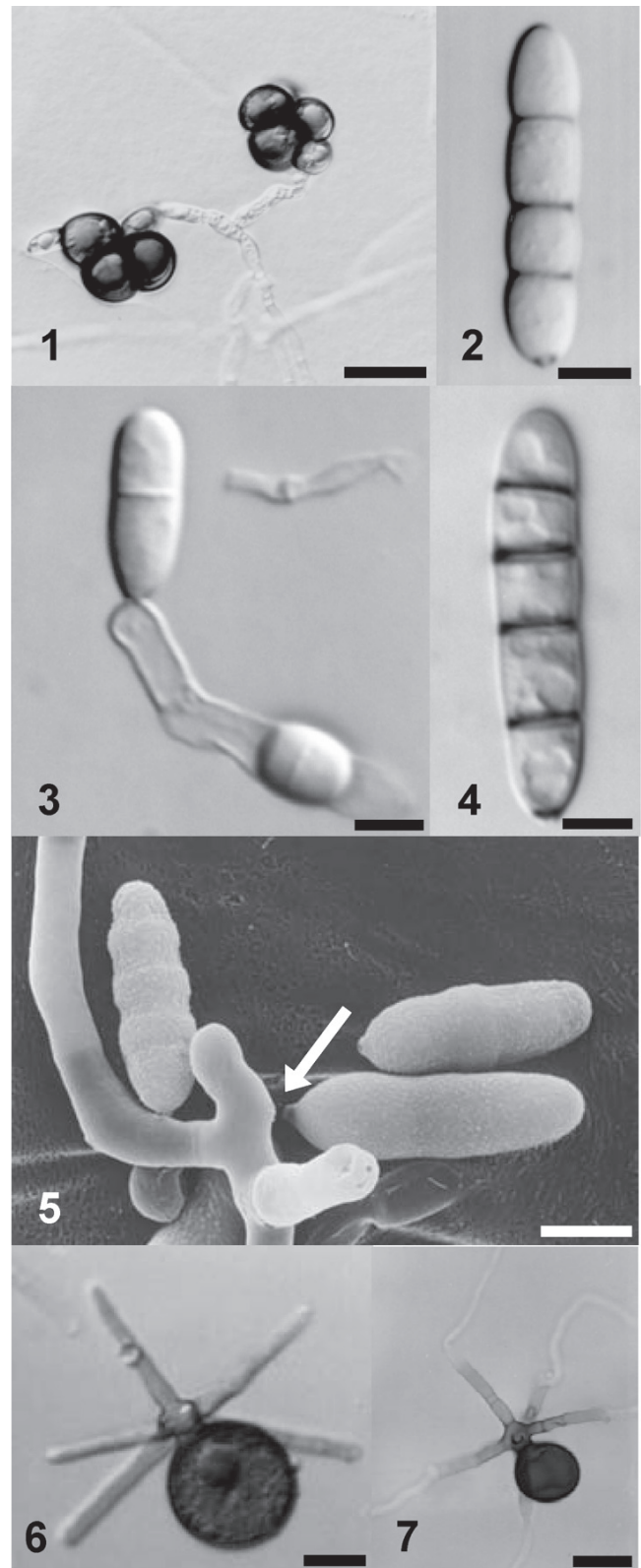
Two marine species are described: *Dendryphiella arenaria* and *Dendryphiella salina* (Figs. 2–5), the former has smaller conidia and fewer septa ($9\text{--}20 \times 3.5\text{--}6.5 \mu\text{m}$, 1–3 septa) while the latter has larger conidia ($14.5\text{--}75.0 \times 5.5\text{--}10.5 \mu\text{m}$, 3–5 septa) (Kohlmeyer & Kohlmeyer, 1979). Conidial measurements vary as can be seen from the following data: *Dendryphiella arenaria* $8\text{--}25 \times 5\text{--}7 \mu\text{m}$ (Ellis, 1976), $16.0\text{--}28.8 \times 3.2\text{--}9.6 \mu\text{m}$ (isolate used in this study); and *Dendryphiella salina* $16\text{--}65 \times 5\text{--}9 \mu\text{m}$ (Ellis, 1976), $13.7\text{--}60.7 \times 3.9\text{--}7.9 \mu\text{m}$ (isolate PP0503 in this study). They have been generally isolated in temperate habitats; *Dendryphiella arenaria* has been found associated with sand, while *Dendryphiella salina* often occurs on decaying seaweed and sea grasses.

The generic assignment of marine *Dendryphiella* species has been confusing since Ellis (1976) transferred these two species to the genus *Scolecobasidium*, which is characterized by polyblastic denticulate conidiogenous cells, leaving cylindrical denticles when conidia become detached (Ellis, 1976). However, this feature has not been observed in *Dendryphiella arenaria* and *Dendryphiella salina* (Kohlmeyer & Kohlmeyer, 1979), or in the description of the type material of *Dendryphiella arenaria* (Nicot, 1958). In the examination of many isolates of *D. salina*, the senior author has never seen denticles on the conidiophores, however prominent scars are visible. This aspect is considered further in the discussion.

Orbimyces spectabilis was described by Barghoorn & Linder (1944) on wood from Charleston, Massachusetts, USA, with a large black basal cell and a crown of 4–5 radiating arms, one polar and 4 latterly placed (Figs. 6–7). This conidial configuration leads to entrapment and colonization of new substrata (Jones, 1994). Germination of the conidia is from the tips of the arms, and never from the basal cell.

Currently two marine anamorphic fungi have their teleomorphs in the Lulworthiales: *Zalerion maritimum* with *Lulworthia uniseptata* as the teleomorph and *Anguillospora marina* with *Lindra obtusa* as the teleomorph. The genus *Zalerion* is not monophyletic as isolates of *Zalerion arboricola* (a terrestrial species) are not congeneric with the type species *Zalerion maritimum*. Consequently the isolate ATCC20868 was transferred to a new genus *Glarea lozoyensis*, based on DNA fingerprinting (Bills et al., 1999). *Zalerion varium* likewise is not congeneric with *Zalerion maritimum*, but groups with *Glarea lozoyensis* in the Leotiaceae, Leotiales (Bills et al., 1999).

The objectives of this paper are to: (1) link *Cumulospora varia*, *Dendryphiella arenaria*, *Dendryphiella salina* and *Orbimyces spectabilis* to their respective teleomorphs by phylogenetic analyses of the nuclear SSU, LSU and ITS rRNA genes, and (2) comment on the nomenclatural position of *Dendryphiella* species and their assignment by Ellis (1976) to *Scolecobasidium*.



Figs. 1–7. Light and scanning electron micrographs of *Cumulospora varia*, *Dendryphiella arenaria*, *D. salina* and *Orbimyces spectabilis*: 1. Conidia of *Cumulospora varia*; 2–3. Conidia of *Dendryphiella arenaria*; 4–5. Conidia of *Dendryphiella salina*. Peg-like extension of the conidium, arrowed in 5; 6–7. Conidia of *Orbimyces spectabilis*. Germinating conidium from the tips of the radiating arms in 7. Scale bars: 1 = 10 μm ; 2–3 = 2 μm ; 4 = 5 μm ; 5 = 10 μm ; 6 = 10 μm , 7 = 20 μm .

MATERIALS AND METHODS

Fungal cultures. – Fungi used in this study are two isolates of *Cumulospora varia* (GR78, IT152), *Dendryphiella arenaria*, two isolates of *Dendryphiella salina* (CY3139, CY3140), *Dendryphiella vinosa* and *Orbimyces spectabilis*. Fungi were isolated from submerged wood and attached mangrove wood collected at various locations (Denmark, England, Thailand). Fungi were maintained on seawater cornmeal agar (Difco). Cultures were grown in GYP broth (4g/L glucose, 4g/L yeast extract, 2 g/L peptone) in filtered seawater, except *D. vinosa* which was grown in a fresh water medium. Fungal biomass was harvested by conventional filtration and washed with sterile distilled water.

DNA extraction, amplification and sequencing. – Approximately 100 mg of frozen mycelium was ground to a fine powder with a pestle and mortar and transferred to a 1.5 ml tube. The ground mycelium was resuspended in 0.4 ml CTAB extraction buffer (100 mM Tris-HCl pH 8.4, 1.4 M NaCl, 25 mM EDTA, 2% CTAB) and incubated at 70°C for 30 min. Following extraction, an equal volume of chloroform was added to each tube, briefly vortexed and centrifuged for 10 min at 12,300 g. The upper phase was removed to a new tube and DNA precipitated by addition of 0.6 ml of chilled isopropanol. After pelleting the DNA at 12,300 g for 5 min, the supernatant was discarded and the pellet gently washed with 70% ethanol and resuspended in 0.1 ml TE buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0).

The nuclear SSU rRNA gene was amplified from genomic DNA using the primers NS1/NS8 (or /NS4) (White et al., 1990). The nuclear LSU rRNA gene was amplified from genomic DNA using JS1/JS8 (or /LR7) (Bunyard et al., 1994; Landvik, 1996). ITS regions of *Dendryphiella arenaria* and *Dendryphiella salina* were amplified by ITS5/ITS4R (White et al., 1990). All genes were amplified using FINNZYMES, DyNAzyme™ II DNA Polymerase Kit (MACHEREY-NAGEL, Product code F-551S), in a Perkin Elmer thermal cycler. The amplification cycle consisted of an initial denaturation step of 94°C for 5 min followed by 35 cycle of (i) denaturation (94°C for 1 min) (ii) annealing (55°C for 1.5 min) and (iii) elongation (72°C for 2.5 min) and a final 10 min elongation step at 72°C. The PCR products were analysed by agarose gel electrophoresis and purified using a NucleoSpin[®] Plant DNA Purification Kit (MACHEREY-NAGEL, Catalogue No. 740 570. 50).

Direct sequencing of purified PCR products was performed using NS1, NS2, NS3, NS4, NS5 and NS8 for SSU rDNA (White et al., 1990), JS1, JS5, JS8 and LR7 for LSU rDNA (Landvik, 1996) and ITS5 and ITS4R for ITS regions (White et al., 1990) with an ABI PRISM Rhodamine Terminator Cycle Sequencing Kit (Applied Biosystem). Reactions and programs were chosen according to the manufacturer's recommendations. Samples were analysed automatically by the personnel at BIOTEC Service Unit (BSU).

Sequence analysis. – After sequence assembly, sequences were aligned manually in Se-Al v1.0a1 with other sequences

obtained from GenBank (Rambaut, 1999). For *Dendryphiella*, two aligned datasets, SSU+LSU and ITS, were analysed in PAUP*4.0b10 (Swofford, 2002). Maximum parsimony was performed initially on the SSU+LSU dataset of 43 taxa using heuristic search (100 replicates of random sequence addition, tree-bisection-reconnection (TBR) branch-swapping). The most parsimonious trees (MPT) obtained were employed for a TBR branch-swapping using maximum likelihood criterion. A thousand bootstrapping analysis using maximum parsimony criterion were calculated through full heuristic (10 replicates of random sequence addition, TBR). Neighbour-joining and bootstrapping analyses were performed on the ITS dataset of 19 taxa using the Jukes-Cantor estimate.

For *Cumulospora varia* and *Orbimyces spectabilis*, a maximum parsimony analysis of a combined dataset of the SSU and LSU rDNA was performed in PAUP*4.0b10 (Swofford, 2002). Initial heuristic searches were run with the following settings: gaps treated as missing data, starting tree(s) obtained via stepwise addition, random sequence addition of 10,000 replicas, a tree-bisection-reconnection (TBR) branch-swapping algorithm, MULTREES off. A thousand parsimony analysis were used to reflect the support of the clades with the same settings with the exception that 10 replicas of random sequence addition were used. In parallel, Bayesian analyses were performed on the dataset in MrBayes v3.1.2: GTR+I+ model, 2 million generations in 4 chains with sampling every 100 generations, discarding the first 25% of the trees (Ronquist & Huelsenbeck, 2003; Huelsenbeck & Ronquist, 2005).

RESULTS AND DISCUSSION

Dendryphiella. – Results from a BLAST search showed that the closest sequence matches in the GenBank to SSU and LSU rDNA sequences of *Dendryphiella arenaria* and *Dendryphiella salina* (CY3139, CY3140) were *Pleospora herbarum*, *Setosphaeria monoceras*, *Cochliobolus* spp. and *Curvularia brachyspora* while those of *Dendryphiella vinosa* were *Cheiromoniliophora elegans*, *Pseudodictyosporium wauense* and *Bimuria novae-zelandiae*. These close sequence matches were incorporated in the phylogenetic analysis along with other sequences in the GenBank. A sequence of *Scolecobasidium* sp. (U20513) was also included due to the fact that marine *Dendryphiella* species were once regarded as *Scolecobasidium* spp. (Ellis, 1976).

Two hundred and sixteen MPTs were obtained from maximum parsimony analysis of a total of 465 parsimony-informative characters with a tree length of 1613 steps, a C.I. of 0.593 and a R.I. of 0.771. One tree with a -ln likelihood score of 14434.49050 was calculated as the best tree by the Kishino-Hesagawa test. An identical tree (-ln likelihood score = 14434.49050) was obtained after a maximum likelihood analysis using the MPTs as the starting trees for branch-swapping and is shown in Fig. 8.

With *Dendryphiella vinosa* residing in the Melanommataceae (Pleosporales), marine *Dendryphiella* (*Dendryphiella arenaria*

and *Dendryphiella salina*) are monophyletic and belong in the Pleosporaceae (Pleosporales). *Dendryphiella vinosa* is in a well-supported clade (98%) with *Dictyosporium elegans*, *Cheimoniliophora elegans* and *Pseudodictyosporium wauense*. On the other hand, *Dendryphiella arenaria* and *Dendryphiella salina* grouped with *Clathrospora diplospora*, *Cochliobolus heterostrophus*, *Curvularia brachyspora*, *Decorospora gaudefroyi*, *Lewia infectoria*, *Pleospora herbarum*, *Pyrenophora trichostoma*, *Pyrenophora tritici-repentis*, *Scolecobasidium* sp. and *Setosphaeria monoceras* with good support (100%). However, bootstrap values on some of the branches in this group are weak, i.e. relationships between these taxa are not resolved. In order to relate the marine *Dendryphiella* to species in the Pleosporaceae, their ITS rDNA was sequenced and compared with other available sequences in the family.

Closest sequence matches to ITS sequences of *Dendryphiella arenaria* and *Dendryphiella salina* (CY3139, CY3140) were *Stemphylium* spp. and *Pleospora herbarum* while those of *Dendryphiella vinosa* were *Dictyosporium* spp. As shown from the SSU+LSU tree, *Dendryphiella vinosa* is distantly

related to *D. arenaria* and *D. salina*. ITS sequence comparison of the three *Dendryphiella* species also revealed that they are very different (results not shown). Therefore, *Dendryphiella vinosa* was not included in the analysis of the ITS dataset. Although combined SSU and LSU rDNA sequence analysis demonstrated that marine *Dendryphiella* species are closely related to a *Scolecobasidium* sp., *Curvularia brachyspora*, *Cochliobolus heterostrophus*, their ITS sequences cannot be aligned unambiguously. Based on BLAST search results and phylogenetic analysis of the combined SSU and LSU dataset, mainly *Pleospora* spp. and *Stemphylium* spp. were included in the neighbour-joining analysis of the ITS rDNA region using *Cochliobolus* spp. as the outgroup taxa.

The tree from the neighbour-joining analysis of the ITS rDNA region is shown in Fig. 9. The teleomorphs of *Dendryphiella arenaria* and *Dendryphiella salina* belong in the Pleosporaceae but it is not known which teleomorphic species they are close to. *Dendryphiella arenaria* and *Dendryphiella salina* are monophyletic in the ITS tree with a moderate bootstrap value (70%). *Stemphylium globuliferum* is closely related to this group but it is not supported by

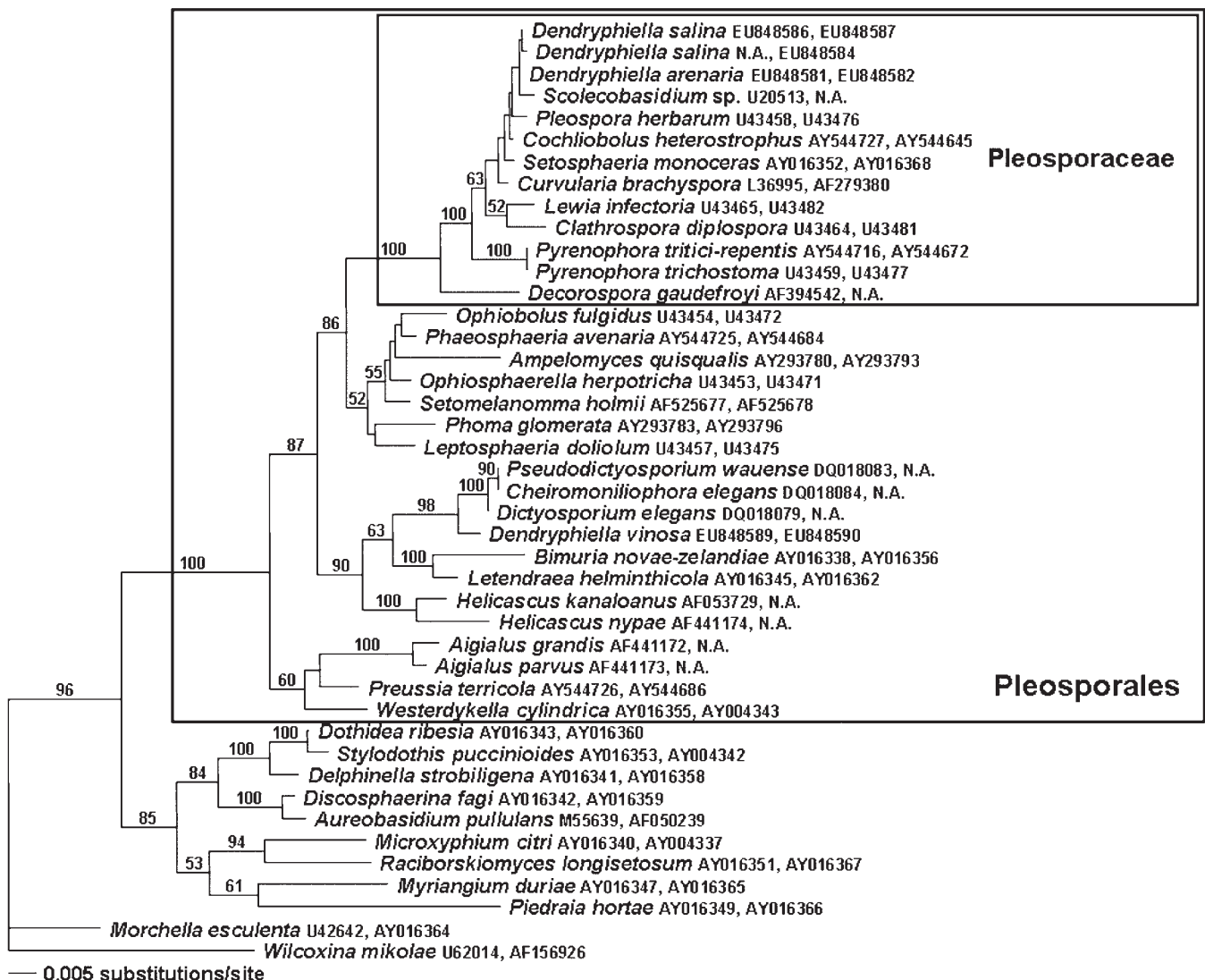


Fig. 8. A maximum likelihood tree with an $-\ln$ likelihood score of 14434.49050 based on the combined SSU and LSU analysis. Parsimony bootstrap values are shown on the branches.

bootstrap. The closest teleomorphic species is *Pleospora triglochinicola*, which is the teleomorph of *Stemphylium triglochinicola*. *Decorospora gaudefroyi* (a marine species) was not included in the analysis as its ITS sequence is very different from that of *Dendryphiella arenaria* and *Dendryphiella salina* (Results not shown).

Cumulospora varia and Orbimyces spectabilis. – Eight thousand, one hundred and seven MPTs were obtained from

maximum parsimony analysis of a total of 679 parsimony-informative characters of SSU+LSU dataset, with a tree length of 1932 steps, a C.I. of 0.636 and a R.I. of 0.774. The consensus tree of the 8,107 MPTs is shown in Fig. 10.

Cumulospora varia and *Orbimyces spectabilis* are well placed in the Lulworthiales (100% bootstrap value and 1.00 posterior probability). The two isolates of *Cumulospora varia*, isolated from Mu Ko Chang Island, Thailand, from different

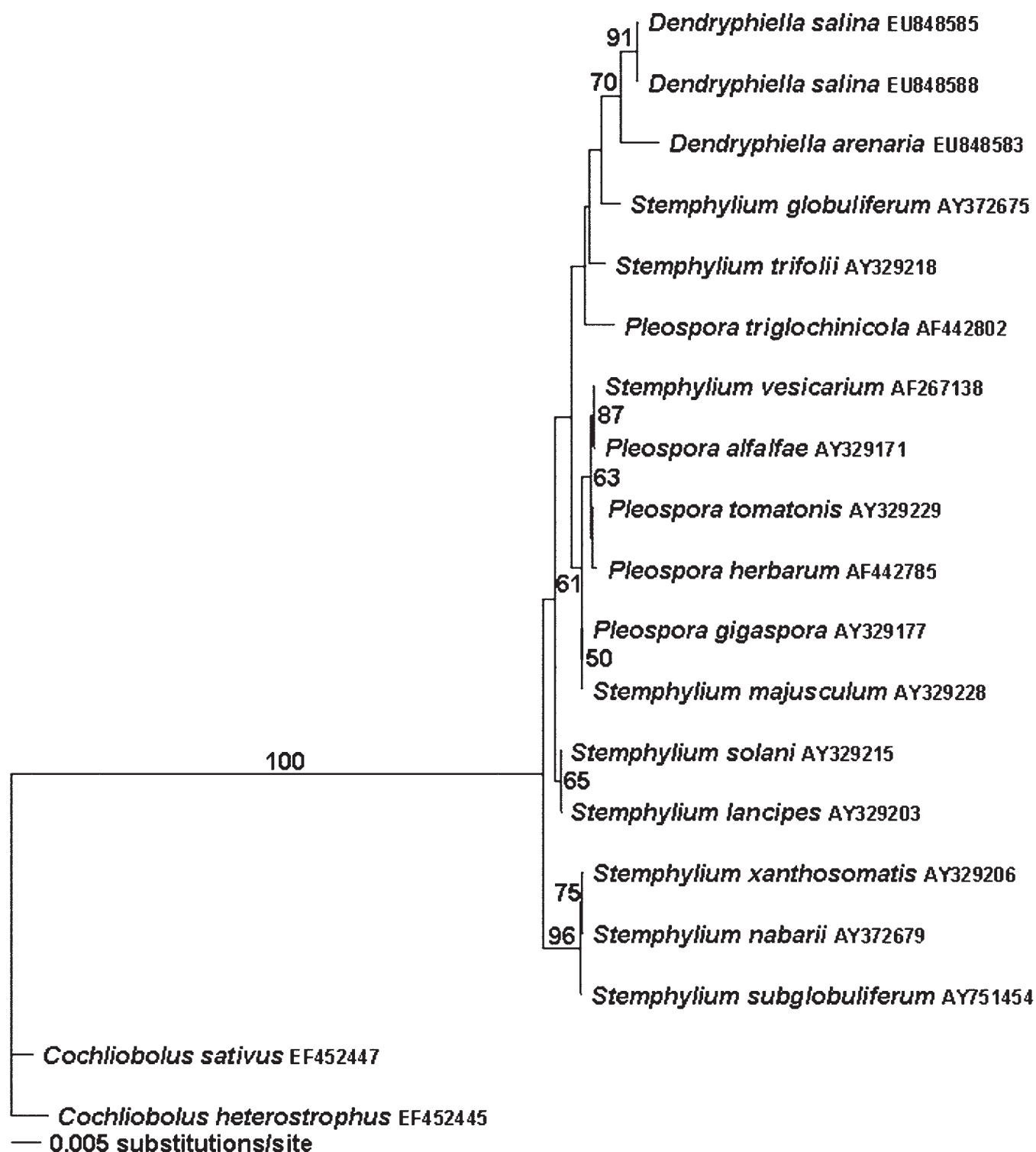


Fig. 9. A neighbour-joining tree using the Jukes-Cantor estimate obtained from ITS rDNA analysis. Bootstrap values by neighbour-joining method are shown on the branches.

collections, are monophyletic, and form a sister group to *Lulwoana uniseptata* and its anamorph *Zalerion maritimum*. *Orbimyces spectabilis*, an infrequently collected anamorphic fungus isolated from intertidal wood in Denmark, was basal to the *Lulwoidea* clade, but with weak support.

The order Lulworthiales was erected by Kohlmeyer et al. (2000) for the genera *Lindra* and *Lulworthia*, removed

from the Halosphaeriales, based on molecular studies using the nuclear SSU and LSU rDNA gene, and morphological observations. Subsequently other genera have been transferred to this order: *Haloguignardia* and *Spathulospora*, similarly based on sequence data (Inderbitzin et al., 2004), and *Kohlmeyeriella* (Campbell et al., 2005). More recently the genus *Lulworthia* has been shown to be polyphyletic and new genera have been designated: *Lulwoidea* (for *Lulworthia*

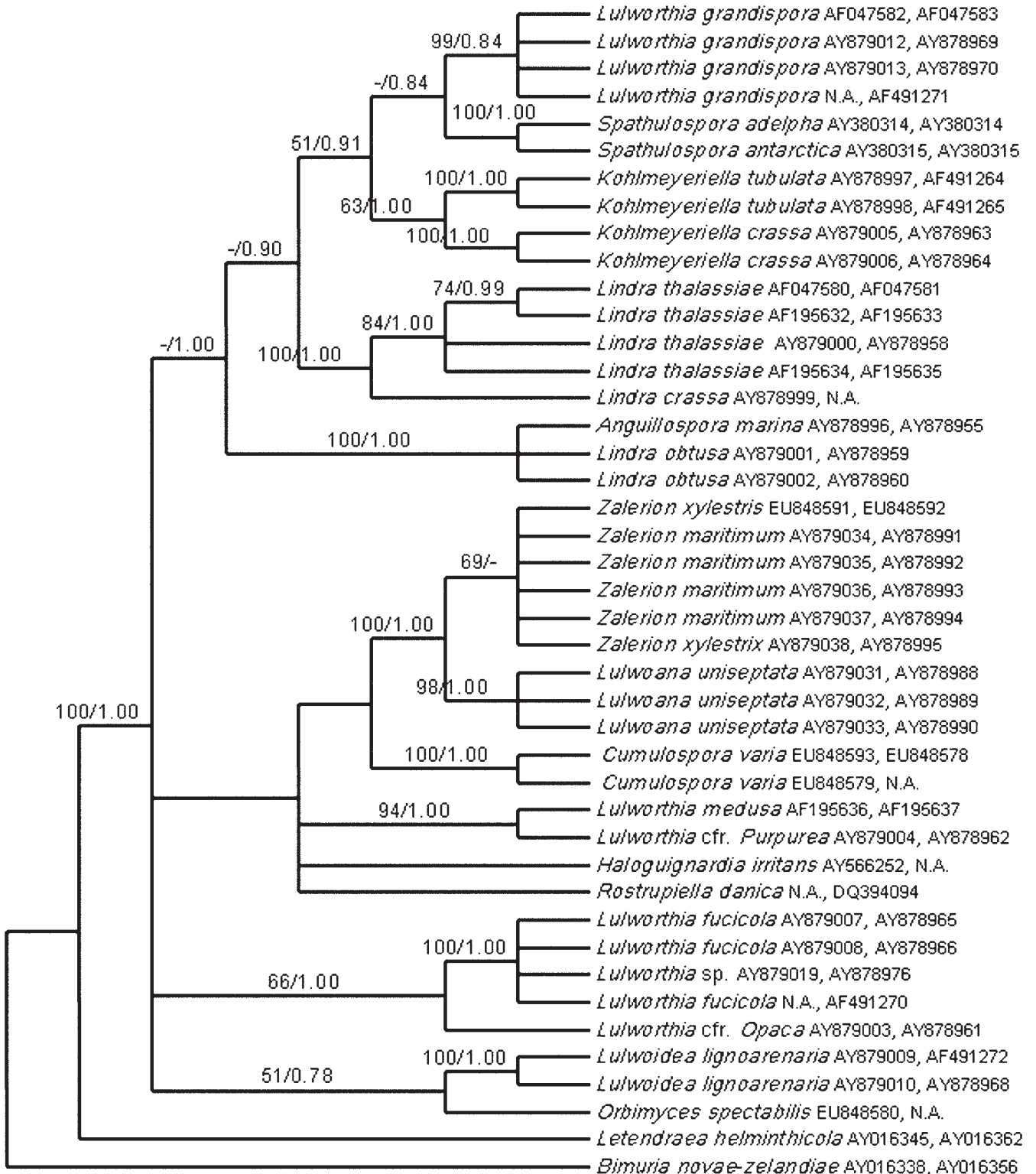


Fig. 10. A consensus tree of 8,107 MPTs inferred from the combined SSU and LSU analysis. Parsimony bootstrap values and posterior probabilities are shown on the branches.

lignoarenaria), *Lulwoana* (for *Lulworthia uniseptata*) (Campbell et al., 2005), and *Rostrupiella* for a new species that did not group with any of the genera listed above (Koch et al., 2007).

These observations indicate that the Lulworthiales is a unique and variable group of usually marine ascomycetes, with only one reported collection of a freshwater *Lulworthia* species (Cai et al., 2002). The anamorphic fungi sequenced here are shown to be closely associated with the order, and indicate its taxonomic complexity. Further sequences of tropical, subtropical and temperate collections of the genus *Lulworthia* are required to examine the linkage with *Cumulospora varia*. Other *Cirrenalia* and *Cumulospora* species need to be sequenced to see if the information obtained is of assistance in the placement of currently unresolved *Lulworthia* species.

CONCLUSIONS

The data presented indicates that the marine *Dendryphiella* species can be classified in the Pleosporaceae, Pleosporales. It is possibly closely related to the genus *Pleospora*. Marine *Dendryphiella* species are shown to be monophyletic but are distantly related to the type species *Dendryphiella vinosa*. Debate continues as to the generic affiliation of the marine *Dendryphiella* species, transferred to *Scolecobasidium* by Ellis (1976), who illustrated denticles on the conidiogenous cells that appear when the conidia become detached. We have not observed these in our light microscope studies or at the scanning electron microscope level (Figs. 2–5). Our observations show a peg-like structure on the conidia of *Dendryphiella salina*, which is extruded from the conidiophore. The extruded peg breaks from the conidiophores, but may remain attached to the conidia after detachment, leaving a scar on the conidiophore. We find no evidence for the transfer of the marine species to *Scolecobasidium* and they are not phylogenetically-related to the type species *Dendryphiella vinosa*. A new genus may be warranted for the two marine *Dendryphiella* species and further taxon sampling is required before this issue can be resolved.

The genera *Cumulospora* and *Orbimyces* can be classified in the Lulworthiales but further studies are required to resolve their relationship with other taxa within the order. Our data also indicate that other genera should be erected for the species currently assigned to the Lulworthiales and not grouping with any of the genera so far delineated (e.g. *Lulworthia* cfr. *purpurea*) (Koch et al., 2007).

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